#1931

CANCER

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
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Application for Research Grant
(Use extra pages as needed)

Date: July 23, 1973

1. Principal Investigator (give title and degrees):

Gerald M. Rosen, Ph.D.; Asst. Professor of Pharmacology

2. Institution & address:

Duke University Medical Center
Durham, North Carolina 27710

3. Department(s) where research will be done or collaboration provided:

Depts. of Physiology & Pharmacology and Biochemistry

4. Short title of study:

The Neoplastic State: Study of the Effects of Irreversible Amino Acid Analogs.

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: December 31, 1975

7. Brief description of specific research aims:

The immediate objective of the proposed research is to first synthesize a series of 3-(p-methoxybenzyl) sydnones with attached alkylating agents since these compounds have been reported to have antineoplastic activity against mouse carcinoma 755. The first phase of the study will then be to test these compounds in vitro utilizing the cell strains presently available in the laboratory. These cells include a rapidly proliferating mammary adenocarcinoma C_3H 2712 tumor and a slow growing mammary adenocarcinoma C_3H BA tumor and a normal mammary gland. The effect of the compound on cellular proliferation, mitotic index, DNA, RNA, and protein synthesis will be used to determine the effect of the drug.

The ultimate purpose of the proposed research is to develop a series of drugs with a potential to selectively destroy neoplastic cells with a minimum of damage to the function of normal cells. Towards this goal, we have the potential utilizing the talents and facilities of both biochemistry and Pharmacology Departments to coordinate information obtained from chemical, biochemical, and pharmacological studies on the effect of these agents in their interaction with macromolecular structures such as DNA and their effect on the synthesis and structure of essential cellular proteins. The potential of this basic information is to provide a number of agents for chemotherapy.

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RESEARCH PLAN

A. Introduction:

1. a. Immediate Objective

The immediate objective of the proposed research is to first synthesize a series of 3-p-(methoxybenzyl) sydnones with attached alkylating agents since these compounds have been reported to have antineoplastic activity against mouse carcinoma 755. The first phase of the study will then be to test these compounds in vitro utilizing the cell strains presently available in the laboratory. These cells include a rapidly proliferating mammary adenocarcinoma C_3H 2712 tumor and a slow growing mammary adenocarcinoma C_3H BA tumor and a normal mammary gland. The effect of the compound on cellular proliferation, mitotic index, DNA, RNA, and protein synthesis will be used to determine the effect of the drug.

b. Ultimate Objectives

The ultimate purpose of the proposed research is to develop a series of drugs with a potential to selectively destroy neoplastic cells with a minimum of damage to the function of normal cells. Towards this goal, we have the potential utilizing the talents and facilities of both Biochemistry and Pharmacology Departments to coordinate information obtained from chemical, biochemical and pharmacological studies. The effect of these agents in their interaction with macromolecular structures such as DNA and their effect on the synthesis and structure of essential cellular proteins will be determined. The potential of this basic information is to provide a number of potential agents for chemotherapy.

2. Background:

a. Chemical

The use of alkylating nitrogen mustards as chemotherapeutic agents in arresting and in a few cases the elimination of cancer had its beginnings during the 2nd world war (1). Since that time, it is unfortunate, however, that only a few of the many thousands of such compounds synthesized through the years have proven to be efficacious (2). It has been proposed that at least part of this problem lies with the stability in situ, of the aziridinium ring. Thus Rosen and Ehrenpreis (3) have shown that the stability of the aziridinium ring is of utmost importance in determining the degree of irreversibility. In the case of nitrogen mustard-antineoplastic agents, for example, the potency of the agent is directly related to its ability to combine covalently, forming an irreversible bond, and thus alter the function of the cell. If the aziridinium ring is too

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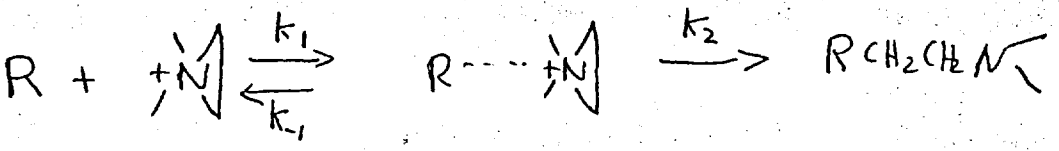
stable, reaction with tissue nucleophiles becomes difficult or impossible and the desired specific irreversible effects of the drug are not realized. By the same token, if the aziridinium ring is insufficiently stabilized, it is too rapidly hydrolyzed to the 2-hydroxyethylamine and the drug will again act only as a reversible agent. Such an explanation has been used to explain the in vivo and in vitro potency of dibenamine and phenoxybenzamine (3) and could readily substantiate similar results with nitrogen mustards. Rosen et al. (3,4,5) have suggested that the aziridinium-aromatic interacting distance has to be sufficient as to allow the aziridinium ring to be stable long enough to permit the drug to diffuse, intact, to the site of action. An example of such a drug although not an antineoplastic agent is the irreversible local anesthetic, 2-[2-chloroethyl)-methylamino]ethyl 4-ethoxybenzoate, whose aziridinium ring has a half life at 30° of 83 minutes (5).

Unfortunately, the lack of specificity of nitrogen mustards has been a major drawback with the use of these agents in cancer chemotherapy. One aspect of this problem as stated above is the stability of the aziridinium ring as compared to its alkylating ability. Furthermore, we have found in our earlier work with local anesthetics, that specificity (in this case, specific alkylation of the receptor sites on the nerve membrane) is related to the ability of the compound to diffuse to the area of interest. Thus there are many factors that are critical in addition to the alkylating moiety to obtain specific pharmacological action. Such is the case of phenoxybenzamine and dibenamine which possess an aziridinium ring of sufficient stability to allow these compounds to alkylate the adrenergic receptor but these agents are without pharmacological effects (3) at the nicotinic receptor of the neuromuscular junction. This phenomenon of specificity has been further explained by the kinetics of irreversible bond formation by dibenamine (6) and local anesthetics (7). Initially, a reversible electrostatic complex is formed between the receptor and the new drug, with time this equilibrium gives way to a non-competitive block, which cannot be reversed within a kinetically measurable time period.



Without this transport concept, indiscriminate alkylation increases to such a degree that statistical distribution of the drug is observed. We propose to overcome much of the problem of the lack of specificity by preparing an agent that not only contains an antitumor moiety but also has a sufficiently stable alkylating pharmacophore. This series of compounds will be discussed in depth later.

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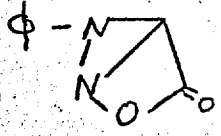
Earl and Mackney (8), working at the University of Sydney, found that treating N-nitroso-N-phenylglycine with acetic anhydride gave a fused ring (I) which they called "sydnone". In 1949, Baker et al, (9) found that the term fused ring was misleading and suggested that structure (II) was more appliable. At the same time Simpson (10) determined that "sydnone" was a charged species although totally neutral. Since the late 1940's, much work has been done on this unusual ring system, with the determination that a class of compounds called "sydnones" is aromatic in nature. The earliest reported consideration of sydnones as biologically important agents was made by Brookes and Walker (11), when they prepared several 3-methyl-4-alkyl-sydnones as potential amino acid antagonists. A number of sydnones have been examined for possible antitumor activity (12). Greco et al. (13) reported activity against carcinoma 755 in mice with 3-(p-methoxybenzyl) sydnone. Under the right specific circumstances the potential use of sydnones as antineoplastic agents, may be enhanced by their similarity in structure to amino acids.



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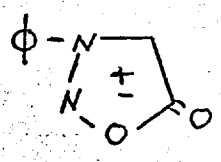

II

b. Biochemical Review of Alkylating Agents

The alkylating agents were the first group of synthetic compounds recognized for their anti-cancer properties. During World War I the bone marrow depressant effects of sulfa mustard bis (β-chlorethyl) sulfide, was noted but ignored. The revival of investigation in potential was gases during the Second World War included among other compounds the nitrogen mustards. In these studies the bone marrow depressant action of one of these agents, tris, (β-chlorethyl) amine hydrochloride suggested its potential value in the treatment of neoplastic diseases of the blood forming organs. The first clinical studies were begun by Gilman in 1942 (Science 103, 656, 1946). In fact, Rhoads summarized clinical experiences of 160 patients with neoplastic diseases in 1946 (JAMA 131, 656, 1946).

The original derivation of the term alkylating comes from the word alkane and has been defined by Warwick (Cancer Research 23, 1315, 1963) as "those compounds capable of replacing a hydrogen atom in another molecule by an alkyl radical".

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All of the alkylating agents are highly reactive chemical compounds capable of reacting with nucleophilic groups such as amino or sulfhydryl groups. Some of the alkylating agents are so chemically active that they will react with water within a few minutes. For this reason many of the alkylating agents must be stored as dry powder and dissolved immediately before administration.

There are two types of alkylating agents: monofunctional and biofunctional. The monofunctional alkylating agents which have only one active alkylating group are far less active in their anti-tumor activity than those containing two alkylating groups. The increased reactivity of bifunctional alkylating agents might have its explanation in that alkylating agents act by binding to DNA (Lawley and Brookes, Nature 206, 480, 1965).

The evidence that alkylating agents may have their primary function in the interstrand binding of double stranded DNA molecules is strengthened by the following observations:

1) Alkylating agents are both mutogenic and carcinogenic.

2) Both in vivo and in vitro alkylating agents produce fragmentation and clumping of chromosomes.

3) Alkylating agents inactivate DNA containing viruses much more readily than RNA viruses.

4) Alkylating agents do not inhibit bacteriophage containing a single stranded DNA although they are effective in alkylating DNA of the host which is double stranded DNA.

5) Alkylating agents are relatively ineffective inhibitors of protein function although they readily inhibit mitosis.

6) There is evidence of interstrand binding from the N7 in the guanine of one strand to the N7 of guanine in the opposite strand and that this interstrand binding prevents the separation of the two strands of DNA in the double coiled helix that is necessary for cell replication.

7) di (guanine-7-yl) derivatives of sulfa mustards have been isolated when this agent is reacted with DNA. Thus these observations would explain why the monofunctional alkylating agents are less effective than bifunctional agents.

It is on the basis of these observations that one could conclude that the efficacy of the alkylating agent is dependent

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on 1) reactivity of the alkylating groups as stated in our proposal and 2) the nature of the side chain that can be specifically designed to interact with the DNA molecule. The fact that alkylating agents are also mutogenic lends convincing support to their primary action at the level of the DNA molecule, as may be seen in recent reviews on alkylating agents: (Cline, Cancer Chemotherapy, Saunders Corp. 1971); (Clark, Cumley, McCay, and Copeland, Oncology, 1970, vol. 2, Experimental Cancer Chemotherapy, Yearbook, Medical publishers, 1970).

3. Rationale

As suggested in the Objective section, the study of the mode of action of sydnone nitrogen mustards on cancerogenic cells by means of refined pharmacological, biochemical and chemical techniques leads to an integrated concept of the characteristics of the neoplastic state.

As stated in the Background section, few of the many thousand nitrogen mustard compounds have been useful as antineoplastic agents. This is due in part to the variable stability of the, in situ, aziridinium ring. In addition, there appears to be no significant pharmacological structure that one can correlate to activity. It has been known that planar molecules might be able to "fit" into the DNA macromolecular structure and thus alter the programming process. The choice of sydnone nitrogen mustards as possible therapeutic agents is based on several pieces of evidence: Firstly, the sydnone ring is planar and in addition it has been used as amino acid analogs. The fact that the sydnone is planar would increase its capacity for intercalation into DNA and in addition as an amino analogue it has the potential to augment its transport. Secondly, Rosen et al, (3,4,5) have determined a possible aromatic-aziridinium distance that allows the ring to be sufficiently stable so that indescriminate aklylation is diminished greatly, which leads to a decrease in toxicity. By the techniques discussed in the Methods of Procedure section, known biochemical techniques can determine the site of action of these drugs.

B. Specific Aims:

Our immediate objective is to determine the effect of sydnone nitrogen mustards on normal and malignant cells in vivo and in vitro.

In the event of their efficacy, we will apply for another grant broadening out studies.

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C. Methods of Procedure:

a. Biochemical Studies

The initial biochemical investigations of the alkylating agents will first include a determination of the LD50 utilizing both C57 and C3H mice from Bar Harbor Jackson Memorial Laboratories. Two types of tumors will be studied in these mice. One represents a fastgrowing spontaneous adenocarcinoma H2712 (Dunham, L. C. and Steward, H. L., 1953, J. of the National Cancer Inst. 13, 1299). This tumor is usually palpable within three days, can be transplanted in ten days and kills the host between 9 and 16 days, and is therefore considered a very fastgrowing tumor. The slowgrowing tumor C3HBA tumor (reference is same as for the H2712) is also a spontaneous tumor of the mammary gland in C3H/An mice. The tumor is usually palpable within six days, is transplantable within 10 days and kills the host in 3 to 7 weeks.

Cells from these animals have been established in tissue culture in Waymouth media MAB87/3 (Charity Waymouth, Cultivation of Cells and Chemically Defined Media and the Malignant Transformation of Cells in vitro, W. Junk, Publishers, The Hague 1965). Most of our studies will utilize both tissue and cell culture in that they are most amenable to study the effect of small quantities of the compound that would not be subjected to biochemical alterations frequently found in vivo. In addition to these cells normal chick embryo fibroblasts and transformed fibroblasts obtained from the laboratory of Dr. Ralph Smith will be used to compare normal euploid cells and cells that have been transformed by virus.

One of the readily identifiable characteristics of normal euploid cells is that represented by contact inhibition as first described by Eagle and later amplified by a large number of other investigators (Cancer Research 29, 1770, 1969). The characteristics of this contact inhibition represent an alteration in gene expression of the normal cell to inhibit the replication and the transcription of its DNA, the result of which is the cessation of cell division and motility of the normal cell population. In contrast, cell cultures obtained from tumors, or cells that have undergone transformations no longer exhibit this characteristic of contact inhibition. We propose then to utilize these characteristics as a preliminary identification of the alteration of a normal to a malignant culture in vitro, and to determine the effect of our synthesized compounds on their ability to regain the capacity of contact inhibition exhibited by normal cells.

All of our studies will include determination of total DNA followed by the method of Ceriotti, G. (J.B.C. 214, 59, 1955) as

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modofied by Short, et al., (Short, E., Warner, H. R. and Koerner, J., J.B.C. 243, 3342, 1968). DNA synthesis will be followed by tritiated thymidine uptake using the procedures in our laboratory and protein synthesis followed by the utilization of C^{14} labelled leucine and RNA synthesis followed using labelled uracyl. Total protein will be determined by the method of Lowry et al., (J.B.C., 193, 265, 1951). The original procedure will be used for the determination of RNA (Ceriotti, J.B.C., 214, 59, 1955).

It is reasonable to assume that an antineoplastic agent will either specifically interact with the cell membrane of the malignant cell and thereby alter its permeability and/or will interact with the DNA by intercalation and alkylation. It is also reasonable to assume that the intercalation into the DNA would require a planar ring and that alkylation could be accomplished by a nitrogen mustard type side chain.

Thus the intercalation model of Lerman (Lerman, L. F., J. Molecular Biology, 3, 18, 1961) has been widely accepted as the mode of strong (primary) interaction between acridines and DNA (Waring, J. Mol. Biol. 54, 247, 1970) and has led to a plausible hypothesis for the mechanism by which acridines produce frame shift mutants. Recently a great deal of evidence has also accumulated that the phenomenon of intercalation may extend to a much wider range of drugs than just the acridines. Thus in addition to carcinogens, tranquilizers and ballucinogens, a number of chemotherapeutic agents may also have their mode of action by intercalation (Waring, J. Mol. Biol. 54, 247, 1970).

As a diagnostic feature of the intercalation process, we will use the technique of drug induced local uncoiling of the double helix of a $\Phi\chi$147 RD DNA. In this system the uncoiling results in the removal and reversal of supercoils of closed circular DNA revealed by changes in sedimentation coefficient (Waring, J. Mol. Biol. 54, 247, 1970). It should also be pointed out that the conversion from a plectonemic form (supercoil) to its paronemic form (open circle) may also be accomplished by the introduction of a single nick in one of the strands (Waring, J. Mol. Biol. 54, 247, 1970). Thus, in addition we will isolate the DNA by phenol procedures (Methods in Enzymology 12, Part A, B, and D, 1968, 1969 and 1971) and fractionate the DNA on cesium chloride isopycnic gradients, (Methods in Enzymology, 12 and 21), and examine it on alkaline sucrose gradients to determine if nicks have resulted from the alkylating sydnone.

b. Preparation of Nitrogen Mustart Sydnones

For simplicity, I will use structures where appropriate in discussing the development of these compounds. The synthetic

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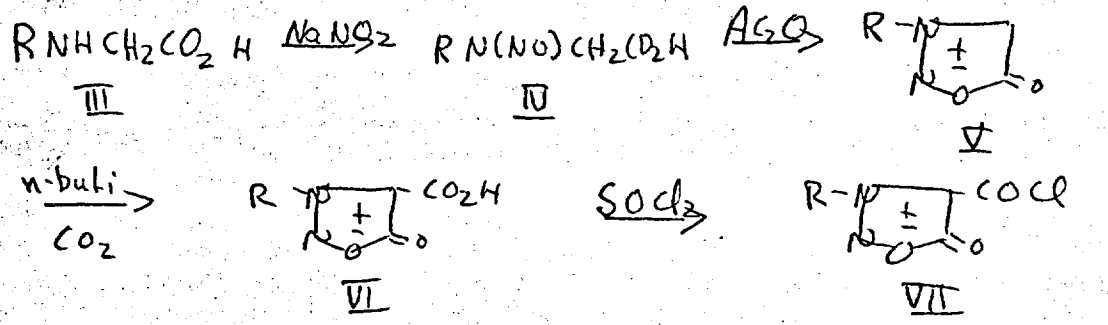
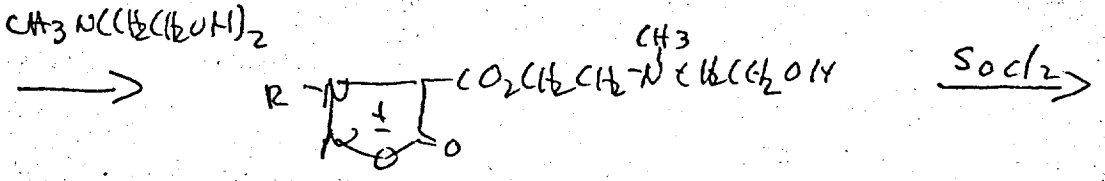
sequence used in the preparation of the sydnone ring system (V) is well documented in the papers of Earl and Mackney (8) and Greco, et al. (13). Greco, Pesce and Franco (14) prepared 3-phenylsydnone-4-carboxylic acid (where R = Phenyl, VI) by the reaction of 4-lithium-3-phenyl-sydnone with carbon dioxide. Conversion to the acid chloride (where R = Phenyl, VII) using thionyl chloride gives a white solid (15). The remaining steps in the development of the sydnone nitrogen (IX) mustard have been discussed by Rosen, et al. (5).

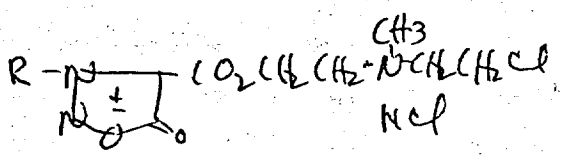


III IV V

VI VII



VIII



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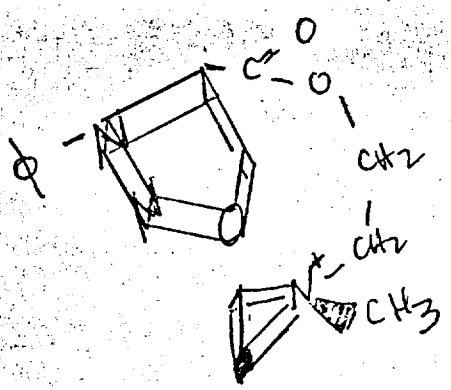
As suggested by the structure below, there is a possibility for the aziridinium ring to be stabilized against rapid hydrolysis by the interaction of the "banana bonding" electrons of the aziridinium ring and the aromatic electrons of the sydnone nucleus. This type of interaction has been suggested (16,5) to account for the increased stability of the aziridinium ring in the irreversible local anesthetic, 2-[chloroethyl)methylamino]ethyl 4-ethoxybenzoate.



D. Significance

There will be two immediate contributions from the proposed research: 1) The effect of sydnone-nitrogen mustards will be clarified. 2) By the use of this irreversible agent, our understanding of the biochemical processes underlying cancerogenic growth may be better understood. These accomplishments hopefully will be of significance in the following manner:

1) Identification of the site of action of sydnone nitrogen mustards would provide us with a better understanding of the mechanism of cancerogenic growth.

2) One or more of the compounds could prove of great value therapeutically for the purposes of arresting and/or eliminating cancer.

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References

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

BIOCHEMISTRY:

Approximately 2,700 square feet of laboratory space is in use for the biochemical investigations in the laboratory of Dr. McCarty. Facilities include all heavy equipment required for the project, 2 Spinco ultracentrifuges, 2 amino acid analyzers, 2 Gilford Spectrophotometers, 1 packard Scintillation Spectrometer, 2 Sorvall centrifuges and continuous flow equipment.

PHARMACOLOGY:

Approximately 1,500 square feet of laboratory space is in use for the chemical and pharmacological investigations in the laboratory of Dr. Rosen, which does not include cold room, animal quarters, and secretarial offices. Facilities include equipment needed to synthesize and test the sydnones. The new equipment is needed to replace those instruments borrowed from Dr. T. Narahashi which include: a pH meter, two balances.

11. Additional facilities required:

The chemistry Department has a N.M.R. spectrometer which is available for my use.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

note additional sheets

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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